15N-labeled dietary nitrate supplementation increases human skeletal muscle nitrate concentration and improves muscle torque production

Stefan Kadach¹ | Ji Won Park² | Zdravko Stoyanov¹ | Matthew I. Black¹ | Anni Vanhatalo¹ | Mark Burnley³ | Peter J. Walter⁴ | Hongyi Cai⁴ | Alan N. Schechter² | Barbora Piknova² | Andrew M. Jones¹

¹Faculty of Health and Life Sciences, University of Exeter Medical School, Exeter, UK
²Molecular Medicine Branch, NIDDK, National Institutes of Health, Bethesda, Maryland, USA
³School of Sport, Exercise and Health Sciences, Loughborough University, Loughborough, UK
⁴Clinical Mass Spectrometry Core, NIDDK, National Institutes of Health, Bethesda, Maryland, USA

Correspondence
Andrew M. Jones, Faculty of Health and Life Sciences, University of Exeter Medical School, Exeter, UK.
Email: a.m.jones@exeter.ac.uk

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Abstract
Aim: Dietary nitrate (NO₃⁻) supplementation increases nitric oxide bioavailability and can enhance exercise performance. We investigated the distribution and metabolic fate of ingested NO₃⁻ at rest and during exercise with a focus on skeletal muscle.

Methods: In a randomized, crossover study, 10 healthy volunteers consumed 12.8 mmol 15N-labeled potassium nitrate (K¹⁵NO₃; NIT) or potassium chloride placebo (PLA). Muscle biopsies were taken at baseline, at 1- and 3-h post-supplement ingestion, and immediately following the completion of 60 maximal intermittent contractions of the knee extensors. Muscle, plasma, saliva, and urine samples were analyzed using chemiluminescence to determine absolute [NO₃⁻] and [NO₂⁻], and by mass spectrometry to determine the proportion of NO₃⁻ and NO₂⁻ that was 15N-labeled.

Results: Neither muscle [NO₃⁻] nor [NO₂⁻] were altered by PLA. Following NIT, muscle [NO₃⁻] (but not [NO₂⁻]) was elevated at 1-h (from ~35 to 147 nmol/g, p < 0.001) and 3-h, with almost all of the increase being ¹⁵N-labeled. There was a significant reduction in ¹⁵N-labeled muscle [NO₃⁻] from pre- to post-exercise. Relative to PLA, mean muscle torque production was ~7% greater during the first 18 contractions following NIT. This improvement in torque was correlated with the pre-exercise ¹⁵N-labeled muscle [NO₃⁻] and the magnitude of decline in ¹⁵N-labeled muscle [NO₃⁻] during exercise (r = 0.66 and r = 0.62, respectively; p < 0.01).

Conclusion: This study shows, for the first time, that skeletal muscle rapidly takes up dietary NO₃⁻, the elevated muscle [NO₃⁻] following NO₃⁻ ingestion declines during exercise, and muscle NO₃⁻ dynamics are associated with enhanced torque production during maximal intermittent muscle contractions.
1 | INTRODUCTION

The signaling molecule, nitric oxide (NO), is essential for the maintenance of normal physiological function. The highly reactive nature and relatively short half-life of NO means that sustained provision of this molecule may be compromised if it is not continuously synthesized. Following its production from L-arginine in a reaction catalyzed by the nitric oxide synthase (NOS) enzymes, NO may be oxidized to form the more stable metabolites, nitrite (NO₃⁻) and nitrate (NO₃⁻). NO₂⁻ and NO₃⁻ are now considered to be storage forms of NO since they can be also reduced under appropriate physiological conditions (i.e., low PO₂) to form NO. Tissue and whole body NO homeostasis is regulated via the synergistic relationship between these complementary pathways of oxidation (L-arginine-NOS-NO) and reduction (NO₃⁻-NO₂⁻-NO).

Following dietary NO₃⁻ ingestion or supplementation, several studies have described elevations in [NO₃⁻] and [NO₂⁻] in biological tissues such as saliva, plasma, urine and, most recently, skeletal muscle. The augmentation of NO bioavailability following dietary NO₃⁻ ingestion may have important physiological and therapeutic effects.

At any given time, skeletal muscle [NO₃⁻] reflects the balance between metabolism of NO₃⁻ into other nitrogen-containing species, oxidation of NO produced via NOS into NO₂⁻ and NO₃⁻, and NO₂⁻ and NO₃⁻ exchange between muscle and blood with the latter facilitated by sialin and chloride channels. While muscle [NO₃⁻] has been shown to be elevated following dietary NO₃⁻ supplementation, the extent to which this results from accretion of the exogenously-supplied NO₃⁻, via uptake from the circulation, is uncertain. Determining the proportional contribution of exogenous NO₃⁻ and endogenously-generated NO₃⁻ to total [NO₃⁻] in muscle as well as other tissues, following dietary NO₃⁻ supplementation, would provide important insight into the distribution of ingested NO₃⁻.

While it is unlikely that NOS activity would be altered following acute dietary NO₃⁻ intake, definitive evidence supporting the uptake of ingested NO₃⁻ into skeletal muscle and other tissues is presently lacking.

Recent observations that muscle [NO₃⁻] is higher than blood [NO₃⁻] in both rodents (gluteus maximus and humans (vastus lateralis) has led to speculation that this relatively high muscle [NO₃⁻] may have functional significance. It has been suggested that skeletal muscle serves as a NO₃⁻ “reservoir” that might be drawn upon, via the circulation, to enhance NO bioavailability in other tissues when access to dietary NO₃⁻ is restricted.

Moreover, skeletal muscle possesses the enzymatic machinery required for the reduction of NO₃⁻ and NO₂⁻ to NO (i.e., xanthine oxidoreductase [XO], aldehyde oxidase [AO], mitochondrial amidoxime-reducing component [MARC]). It is, therefore, possible that local muscular NO₃⁻ and/or NO₂⁻ stores are important in muscle function, including the regulation of contractile activity, blood flow distribution and mitochondrial respiration, particularly during exercise when lower PO₂ and pH may favor NO production via the reduction of NO₃⁻ and NO₂⁻. While it has been reported that muscle [NO₃⁻] declines during exercise, at least when it has been elevated by prior dietary NO₃⁻ supplementation, the influence of elevated muscle [NO₃⁻], per se, on exercise performance has not been determined.

The purpose of the present investigation was to employ a stable isotope tracer (K¹⁵NO₃) to determine the distribution and metabolic fate of ingested dietary NO₃⁻ at rest and following exercise. To provide insight into the relative contribution of exogenously-supplied vs endogenously-generated NO₃⁻ on [NO₃⁻] and [NO₂⁻] in skeletal muscle as well as other tissues, we measured the absolute concentrations of NO₃⁻ and NO₂⁻ using chemiluminescence and the percentage of ¹⁵N labeled NO₃⁻ and NO₂⁻ by ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS). We hypothesized that there would be significant increases in skeletal muscle [NO₃⁻] following the ingestion of a K¹⁵NO₃ tracer, with this increase being consequent to the accrual of the labeled NO₃⁻ onto an unchanged basal [NO₃⁻]. We also hypothesized that the elevated muscle [NO₃⁻] would facilitate, and be correlated with, skeletal muscle performance during a maximal exercise task.

2 | RESULTS

2.1 | Distribution of ingested NO₃⁻ at rest

2.1.1 | Skeletal muscle [NO₃⁻] and [NO₂⁻]

Total skeletal muscle [NO₃⁻] was elevated above baseline at 1-h (from 35 ± 9 to 147 ± 71 nmol/g, p < 0.001) before falling slightly but remaining elevated compared to baseline at 3-h following NO₃⁻ ingestion
(105 ± 41 nmol/g, p < 0.001) (Figure 1A). 15N labeled muscle [NO₃⁻] was elevated above baseline at both 1-h (100 ± 63 nmol/g, p < 0.001) and 3-h (50 ± 25 nmol/g, p < 0.001), with the value at 1-h being higher than that at 3-h (p < 0.05). Endogenous (i.e., unlabeled) muscle [NO₃⁻] did not increase above baseline at 1-h (47 ± 16 nmol/g) but was higher than baseline at 3-h (55 ± 21 nmol/g, p < 0.05). There was no change in total muscle [NO₃⁻] following NO₃⁻ ingestion (Figure 1B). 15N labeled muscle [NO₂⁻] was greater than baseline at 1-h (0.12 ± 0.14 nmol/g, p < 0.05), and tended to be higher at 3-h (0.40 ± 0.54 nmol/g, p = 0.05) whereas unlabeled muscle [NO₂⁻] was unchanged.

2.1.2 | Plasma [NO₃⁻] and [NO₂⁻]

Total plasma [NO₃⁻] was elevated above baseline at both 1- and 3-h (from 29 ± 6 to 451 ± 46 and 431 ± 48 nmol/g, respectively, p < 0.001) following NO₃⁻ ingestion, with no difference between 1- and 3-h (Figure 1C). 15N labeled plasma [NO₃⁻] was increased above baseline at 1-h (423 ± 45 nmol/g; p < 0.001) and 3-h (405 ± 45 nmol/g; p < 0.001). Unlabeled plasma [NO₃⁻] was not different from baseline at 1-h (from 28 ± 6 to 28 ± 5 nmol/g) but was lower than baseline at 3-h (27 ± 6 nmol/g; p < 0.05). Total plasma [NO₂⁻] was greater than baseline at 1-h (from 0.13 ± 0.02 to 0.29 ± 0.07 nmol/g; p < 0.001) and 3-h (0.47 ± 0.14 nmol/g; p < 0.001) and the value at 3-h was greater than that at 1-h (p < 0.01) (Figure 1D). 15N labeled [NO₂⁻] was greater than baseline at 1-h (0.08 ± 0.04 nmol/g, p < 0.001) and 3-h (0.21 ± 0.10 nmol/g; p < 0.001) and the value at 3-h was greater than that at 1-h (p < 0.05). Unlabeled plasma [NO₂⁻] was also increased above baseline at 1-h (from 0.12 ± 0.02 to 0.20 ± 0.03 nmol/g; p < 0.001) and 3-h (0.26 ± 0.05 nmol/g; p < 0.001), and the value at 3-h was greater than that at 1-h (p < 0.05).

2.1.3 | Salivary [NO₃⁻] and [NO₂⁻]

Total salivary [NO₃⁻] was increased above baseline at both 1- and 3-h following NO₃⁻ ingestion (Baseline: 194 ± 182; 1-h: 1436 ± 5782; 3-h: 16536 ± 8300 nmol/g, both p < 0.001) and there was no difference between 1- and 3-h (Figure 1E). Both 15N labeled and unlabeled salivary [NO₃⁻] were greater than baseline at 1- and 3-h. Total salivary [NO₂⁻] was increased above baseline at both 1- and 3-h (Baseline: 229 ± 213; 1-h: 2530 ± 1775; 3-h: 3108 ± 1460 nmol/g; both p < 0.001) (Figure 1F). 15N labeled salivary [NO₂⁻] was greater than baseline at both 1-h (p < 0.05) and 3-h (p < 0.001) but there was no change in unlabeled salivary [NO₂⁻] following NO₃⁻ ingestion.

2.1.4 | Urinary [NO₃⁻] and [NO₂⁻]

Total urinary [NO₃⁻] was greater than baseline at 1-h (from 739 ± 225 to 2567 ± 1360 nmol/g, p < 0.05) and 3-h (2466 ± 1070 nmol/g; p < 0.001) following NO₃⁻ ingestion, and there was no difference between 1- and 3-h (Figure 1G). 15N labeled urinary [NO₃⁻] was greater than baseline at 1- and 3-h (p < 0.001) whereas unlabeled urinary [NO₃⁻] was greater at baseline compared to 1-h (p < 0.05) and 3-h (p < 0.001). Total urinary [NO₂⁻] was not different from baseline at either 1- or 3-h (Figure 1H). 15N labeled urinary [NO₂⁻] was greater than baseline at 1-h (p < 0.001) and 3-h (p < 0.05). In contrast, unlabeled urinary [NO₂⁻] was lower than baseline at 1- and 3-h (p < 0.05).

2.1.5 | Plasma/muscle [NO₃⁻] and plasma/muscle [NO₂⁻] ratios

At baseline, muscle [NO₃⁻] was greater than plasma [NO₃⁻] (ratio of 0.8; plasma: 28 ± 6; muscle: 35 ± 9 nmol/g; p < 0.05). Following NO₃⁻ ingestion, the ratio increased to 3.6 at 1-h (plasma: 451 ± 46; muscle: 160 ± 63 nmol/g; p < 0.001) and 4.4 at 3-h (plasma: 431 ± 56; muscle: 111 ± 39 nmol/g; p < 0.001). The plasma/muscle [NO₃⁻] ratio was greater than muscle at both 1-h (p < 0.05) and 3-h (p < 0.001) (Figure 2A). At baseline, plasma [NO₂⁻] was lower than muscle [NO₂⁻] (ratio of 0.18; plasma: 0.13 ± 0.02; muscle: 0.91 ± 0.51 nmol/g; p < 0.05). Following NO₃⁻ ingestion, the ratio was 0.45 at 1-h (plasma: 0.29 ± 0.07; muscle: 0.69 ± 0.23 nmol/g, p < 0.001) and 0.63 at 3-h (plasma: 0.48 ± 0.14; muscle: 0.94 ± 0.48 nmol/g; p < 0.05). The plasma/muscle [NO₂⁻] ratio was greater than baseline at both 1- and 3-h (p < 0.01) (Figure 2B).

2.2 | Metabolic fate of ingested NO₃⁻ during exercise

Compared to placebo, NO₃⁻ ingestion resulted in significant increases in pre-exercise skeletal muscle [NO₃⁻], plasma [NO₂⁻] and [NO₃⁻], and salivary [NO₃⁻] and [NO₂⁻] (p < 0.01).

2.2.1 | Skeletal muscle [NO₃⁻] and [NO₂⁻]

Total muscle [NO₃⁻] did not change from pre-exercise (105 ± 41 nmol/g) to post-exercise either in the control leg (93 ± 35 nmol/g) or the exercised leg (71 ± 40 nmol/g; p = 0.09) in the NIT condition (Figure 3A). Moreover, exercise did not alter total muscle [NO₃⁻] in the PLA condition.
FIGURE 1 Mean ± SD skeletal muscle [NO$_3^-$] (Panel A) and [NO$_2^-$] (Panel B), plasma [NO$_3^-$] (Panel C) and [NO$_2^-$] (Panel D), salivary [NO$_3^-$] (Panel E) and [NO$_2^-$] (Panel F), and urinary [NO$_3^-$] (Panel G) and [NO$_2^-$] (Panel H), prior to (0-h) and at 1-h and 3-h following the ingestion of K$_{15}$NO$_3$ (12.8 mmol NO$_3^-$; ~1300 mg). The height of the bars represent the total concentrations and the proportion of unlabeled and $^{15}$N labeled NO$_3^-$ and NO$_2^-$ is shown in blue and red, respectively. Individual participant responses are shown alongside each panel with total response in black font. $^a$Significant difference compared to 0-h ($p<0.05$). $^b$Significant difference between 1- and 3-h ($p<0.05$). Black, blue, and red letters refer to comparisons between total, unlabeled and $^{15}$N labeled data, respectively.
\[15\]N labeled plasma \([\text{NO}_3^-]\) was lower following exercise \((p < 0.05)\) whereas unlabeled plasma \([\text{NO}_3^-]\) increased slightly following exercise \((p < 0.05)\) in the NIT condition. Total plasma \([\text{NO}_2^-]\) decreased during exercise in the NIT condition \((\text{Pre-exercise: } 0.47 \pm 0.14 \text{ vs. post-exercise: } 0.41 \pm 0.13 \text{nmol/g}; p < 0.05)\) but not in the PLA condition \((\text{Figure 3D})\). There was no change in \([\text{NO}_2^-]\) labeled plasma \([\text{NO}_3^-]\) during exercise in NIT. Compared to pre-exercise baseline, unlabeled plasma \([\text{NO}_3^-]\) was lower following exercise in NIT \((p < 0.001)\) but not in PLA.

2.3 | Exercise performance

The peak MVC torque achieved prior to the 5-min all-out test was 266 ± 95 N/m and 260 ± 60 N/m, and the mean torque sustained during this contraction was 221 ± 83 N/m and 220 ± 49 N/m for PL and NIT, respectively. Voluntary activation of the knee extensors achieved during the MVCs performed prior to the 5-min all-out test was 91 ± 8% and 91 ± 5% for PLA and NIT, respectively. Baseline peak MVC torque, mean torque and voluntary activation were not different between conditions. To facilitate comparisons between PLA and NIT conditions, all torque profiles were subsequently normalized to the MVC values achieved during the PLA condition \((\text{MVCPL})\).

2.3.1 | 5-min test torque profile

The profile for peak torque and mean torque across all participants during each contraction for the 5-min test is shown in Figure 4. During the PLA trial, normalized peak torque declined from 90 ± 9% MVC during the first contraction to 40 ± 10% MVC during the final contraction \((p < 0.001)\). The overall decline in normalized peak torque was not significantly different between the NIT \((-52 \pm 15\%)\) and PLA \((-55 \pm 20\%)\) conditions. There were no significant differences between the PLA and NIT conditions for the overall decline in mean torque or torque impulse across the 60 MVCs, and no differences were observed for CT or impulse above CT \((\text{Table 1})\).

During the first 90-s of the test, participants generated significantly \((p < 0.05)\) higher mean mean torque \%\text{MVCPL} for NIT \((90 \pm 13\%)\) compared to PLA \((83 \pm 6\%)\). Participants also tended to generate a higher mean peak torque \%\text{MVCPL} \((p = 0.05)\) and higher peak mean torque \%\text{MVCPL} \((p = 0.06)\) for NIT compared to PLA \((\text{Figure 4})\).

The potentiated doublet responses and voluntary activation across all participants at each assessment time-point during the 5-min all-out test are provided in Figure 5. During the PLA trial, potentiated doublet responses decreased from 88 ± 11 Nm during the first contraction to

### Figure 2

Mean ± SD plasma/muscle \([\text{NO}_3^-]/[\text{NO}_2^-]\) ratio (Panel A) and plasma/muscle \([\text{NO}_2^-]/[\text{NO}_3^-]\) ratio (Panel B) prior to (0-h) and at 1- and 3-h following the ingestion of a K\(^{15}\)NO\(_3\) supplement.

\(\text{aSignificant difference compared to 0-h (} p < 0.05).\)
40 ± 12 Nm during the final contraction (p < 0.001). This overall decline was not significantly different between PL (−54 ± 15%) and NIT (−55 ± 17%) (p > 0.05). Voluntary activation changed across time (p < 0.05) during the 5-min all-out test (Figure 1B), but there were no differences between PL and NIT.

2.3.2 Relationships between muscle and plasma [NO3\(^{-}\)] and [NO2\(^{-}\)] and exercise performance

At baseline, no significant correlations were observed between total muscle [NO3\(^{-}\)] and muscle [NO2\(^{-}\)] or between total plasma [NO3\(^{-}\)] and plasma [NO2\(^{-}\)] (all P > 0.05). The % increase in total muscle [NO3\(^{-}\)] from baseline to 3-h following NO3\(^{-}\) ingestion was not correlated with baseline total muscle [NO3\(^{-}\)] but was correlated with the % increase in total plasma [NO3\(^{-}\)] (r = 0.77, p < 0.001). The % increase in total muscle [NO3\(^{-}\)] from baseline to 3-h was correlated with the magnitude of its decline during exercise (r = 0.68, p < 0.001). During exercise, the % decrease in total muscle [NO3\(^{-}\)] was correlated with the % decrease in total plasma [NO3\(^{-}\)] (r = 0.52, p < 0.05), and the concomitant % increases in total muscle [NO2\(^{-}\)] (r = 0.46, p < 0.05) and plasma [NO2\(^{-}\)] (r = 0.75, p < 0.001). These % changes in total muscle [NO3\(^{-}\)] were closely related to % changes in 15N labeled muscle [NO3\(^{-}\)] both following NO3\(^{-}\) ingestion (r = 0.92, p < 0.001) and during exercise (r = 0.89, p < 0.001).
Changes in total muscle \([\text{NO}_3^-]\) or \([\text{NO}_2^-]\) either following \(\text{NO}_3^-\) ingestion or during exercise were not correlated with exercise performance variables. However, the pre-exercise \(^{15}\text{N}\) labeled muscle \([\text{NO}_3^-]\) was correlated with peak torque \%MVCPL, mean torque \%MVCPL, peak mean torque \%MVCPL and mean mean torque \%MVCPL during the first 90s of the 5-min test \((r = 0.63-0.69, \text{all } p < 0.01)\). Moreover, a greater absolute decrease in \(^{15}\text{N}\) labeled muscle \([\text{NO}_3^-]\) during exercise was significantly correlated with a greater peak mean torque \%MVCPL \((r = 0.71, p < 0.001)\) and a greater mean mean torque \%MVCPL \((r = 0.62, p < 0.01)\) during the first 90s of the 5-min test. In contrast, there were no significant correlations between changes in total, or \(^{15}\text{N}\) labeled, plasma \([\text{NO}_3^-]\) and \([\text{NO}_2^-]\) during exercise and exercise performance.

3 | DISCUSSION

We used a stable isotope tracer (K\(^{15}\text{NO}_3\)) to determine the distribution of ingested dietary \(\text{NO}_3^-\) at rest and its metabolic fate during exercise. The principal original findings of the present study confirmed our hypotheses by showing that: (1) labeled dietary \(\text{NO}_3^-\) accumulates in skeletal muscle within 1-h of its ingestion, elevating total muscle \([\text{NO}_3^-]\) in the absence of changes in basal (unlabeled) \([\text{NO}_3^-]\); (2) compared to placebo, dietary \(\text{NO}_3^-\) ingestion results in enhanced peak and mean torque production over the first ~90s of a 5-min exercise test involving intermittent isometric MVCs of the knee extensors; and (3) the improved torque production was significantly correlated with both the pre-exercise levels of \(^{15}\text{N}\) labeled muscle \(\text{NO}_3^-\) and the decrease in \(^{15}\text{N}\) labeled muscle \(\text{NO}_3^-\) during exercise. These results show for the first time that acute elevation of muscle \([\text{NO}_3^-]\), achieved via dietary \(\text{NO}_3^-\) ingestion, enhances muscle contractile performance.

3.1 | Distribution of ingested \(\text{NO}_3^-\) at rest

Several previous studies have shown that tissue and body fluid (e.g., skeletal muscle, plasma, saliva, urine) \([\text{NO}_3^-]\)
and/or [NO₂⁻] are increased following dietary NO₃⁻ supplementation and it has been assumed that it is the ingested NO₃⁻ that is directly responsible for this increase. However, NO₃⁻ is also derived from NOS activity and definitive evidence that dietary NO₃⁻, per se, is responsible for the observed increases in tissue [NO₃⁻] and [NO₂⁻] is lacking. In the present study we employed a stable isotope tracer (K¹⁵NO₃) to determine the distribution of ingested dietary NO₃⁻. To achieve this, we used standard chemiluminescence to measure the absolute [NO₃⁻] and [NO₂⁻] in skeletal muscle, plasma, saliva and urine, and we determined the percentage of ¹⁵NO₃⁻ and ¹⁵NO₂⁻ in those same tissues and body fluids using UPLC–MS/MS. Our results show that elevations in tissue and body fluid [NO₃⁻] and [NO₂⁻] following dietary NO₃⁻ supplementation are almost exclusively consequent to the introduction of exogenous NO₃⁻ to the body.

The profiles of changes in [NO₃⁻] above baseline following dietary NO₃⁻ supplementation were broadly similar in muscle, plasma, saliva and urine, with rapid dynamics (i.e., a significant increase within 1-h of NO₃⁻ ingestion) and with [NO₃⁻] remaining elevated at 3-h. These findings are consistent with earlier reports. Interestingly, at 1-h, ¹⁵N labeled NO₃⁻ accounted for ~94%, 96% and 89% of the total NO₃⁻ present in plasma, saliva and urine, respectively, but only ~68% of the total NO₃⁻ in muscle. This is consistent with previous findings of a relatively smaller change in muscle [NO₃⁻] compared to plasma [NO₂⁻] following NO₃⁻ ingestion and may suggest the existence of a barrier to the entry of NO₃⁻ into muscle such as saturation of the sialin transporter or chloride channels.

Similar to our previous study, there were significant increases in total salivary [NO₂⁻] and total plasma [NO₂⁻] but no change in total muscle [NO₂⁻] or total urinary [NO₂⁻] at 1- or 3-h following NO₃⁻ ingestion. The temporal profiles of salivary and plasma [NO₂⁻] we observed are consistent with our understanding of the nitrate-nitrite-NO pathway in which ingested NO₃⁻ enters the enterosalivary circulation, is concentrated by the salivary gland and is then reduced to NO₂⁻ by the oral microbiota before being swallowed and entering the bloodstream, resulting in increased plasma [NO₂⁻]. We found that the increased total salivary [NO₂⁻] was entirely due to increased ¹⁵N labeled [NO₂⁻] whereas the increased total plasma [NO₂⁻] resulted from increases in both labeled and unlabeled [NO₂⁻]. The lack of change in total muscle [NO₂⁻] following NO₃⁻ ingestion is consistent with our previous work. It is noteworthy that these results in humans are in contrast to rodent studies in which muscle [NO₂⁻] is significantly increased by NO₃⁻ ingestion. Possible explanations for these inter-species differences were discussed previously but might also include differences between bolus ingestion (present study) and continuous feeding of NO₃⁻ via drinking water. Despite the lack of change in total muscle [NO₂⁻] in the present study, there was a significant increase in ¹⁵N labeled [NO₂⁻] at 1-h post NO₃⁻ ingestion, which might be due either to local labeled NO₃⁻ reduction or absorption from the circulation.

An intriguing finding in the present study was that total muscle [NO₃⁻] fell by ~29% from 1- to 3-h. This is in contrast to our previous study in which muscle [NO₃⁻] was unchanged over the same time period following the same acute NO₃⁻ ingestion protocol. Moreover, we found that the ¹⁵N labeled fraction of the total muscle [NO₃⁻] fell from 100 to 50 nmol/g, mirrored by a non-significant rise in labeled muscle [NO₂⁻], whereas the unlabeled fraction of the total muscle [NO₃⁻] increased from 34 to 47 nmol/g. These changes over time suggest continued “processing” of the ingested NO₃⁻ within muscle, which might include reduction to NO₂⁻ and other nitrogen-containing metabolites, and potentially some “cross talk” with NOS-mediated NO generation. However, interpretation of these data, along with the finding of an increased unlabeled plasma...
[NO\textsubscript{2}] following \textsuperscript{15}N labeled NO\textsubscript{3} ingestion, is complicated by the possibility of continued movement of labeled and unlabeled NO\textsubscript{3} and NO\textsubscript{2} between the muscle and bloodstream, or vice versa, which we were unable to quantify using the present experimental model.

At baseline, skeletal muscle [NO\textsubscript{3}\textsuperscript{-}] was greater than plasma [NO\textsubscript{3}\textsuperscript{+}] with the plasma/muscle [NO\textsubscript{3}\textsuperscript{-}] ratio being ~0.8. This is in agreement with previous findings in rodents\textsuperscript{12,13} and humans\textsuperscript{14,15,16}. It has been suggested that this NO\textsubscript{3}\textsuperscript{-} concentration gradient between skeletal muscle and plasma enables transport of NO\textsubscript{3} from muscle to support NO requirements in other tissues or organs especially when demand is high or dietary NO\textsubscript{3} intake is restricted\textsuperscript{3,12,18,21}. Following NO\textsubscript{3} ingestion, the plasma/muscle [NO\textsubscript{3}\textsuperscript{-}] ratio increased to 3.6 and 4.4 at 1- and 3-h, respectively, reversing the muscle-to-plasma NO\textsubscript{3} concentration gradient that was evident at baseline. Immediately following NO\textsubscript{3} ingestion, it is possible that the negative plasma-to-muscle NO\textsubscript{3}\textsuperscript{-} gradient mandates that muscle NO\textsubscript{3}\textsuperscript{-} accrual depends on active NO\textsubscript{3}\textsuperscript{-} transport via sialin and/or the chloride channels, whereas when the plasma-to-muscle NO\textsubscript{3}\textsuperscript{-} gradient later becomes positive, muscle NO\textsubscript{3}\textsuperscript{-} uptake from blood may also occur via diffusion. Our results confirm a previous report\textsuperscript{16} that, in humans, muscle [NO\textsubscript{2}\textsuperscript{+}] is considerably greater than plasma [NO\textsubscript{2}\textsuperscript{-}] both in the basal state (plasma/muscle [NO\textsubscript{2}\textsuperscript{-}] ratio of 0.18) and following NO\textsubscript{3} ingestion (plasma/muscle [NO\textsubscript{2}\textsuperscript{-}] ratio of 0.63 at 3-h). This substantial muscle-to-plasma NO\textsubscript{2} concentration gradient in humans differs from that reported in rodents\textsuperscript{12,13} and, while NO\textsubscript{3} is considered more stable and, therefore, better suited to a role as an NO storage molecule\textsuperscript{3}, it is possible that NO\textsubscript{2} is favored for this purpose in humans.\textsuperscript{16}

3.2 Metabolic fate of ingested NO\textsubscript{3} during exercise

We hypothesized that the elevated muscle [NO\textsubscript{3}\textsuperscript{-}] resulting from NO\textsubscript{3} ingestion would facilitate muscle contractile activity during exercise. Previous studies in rats have reported a decline in muscle [NO\textsubscript{3}\textsuperscript{-}] and a rise in muscle [NO\textsubscript{2}\textsuperscript{-}] during exercise, results which might indicate an increased reduction of NO\textsubscript{3} to NO\textsubscript{2} to support NO bioactivity in processes such as vasodilation, mitochondrial respiration and myocyte contractility.\textsuperscript{12,13} It is known that skeletal muscle contains nitrate and nitrite reductases including XO, AO and MARC\textsuperscript{13,15,20} and, therefore, has the necessary “machinery” for NO production via NO\textsubscript{3} and NO\textsubscript{2}. In humans, we previously observed a fall in muscle [NO\textsubscript{3}\textsuperscript{-}] during high-intensity cycle exercise, but only when muscle [NO\textsubscript{3}\textsuperscript{-}] had been elevated by prior NO\textsubscript{3} ingestion.\textsuperscript{15} In the present study, total muscle [NO\textsubscript{3}\textsuperscript{-}] did not fall significantly during exercise when it was preceded by NO\textsubscript{3} ingestion (p = 0.09). It is unknown to what extent differences in exercise modality or muscle contraction regimen (i.e., high-intensity cycling vs. maximal voluntary contractions of the knee extensors) might influence the muscle [NO\textsubscript{3}\textsuperscript{-}] response to exercise.

An important novel observation was that \textsuperscript{15}N labeled muscle [NO\textsubscript{3}\textsuperscript{-}] decreased significantly during exercise whereas there was no change in unlabeled muscle [NO\textsubscript{3}]. This suggests that recently ingested NO\textsubscript{3}, following its arrival at the muscle, might be more “accessible” than the baseline NO\textsubscript{3} store, and raises the intriguing possibility that NO\textsubscript{3} which is generated endogenously and NO\textsubscript{3} which is supplied exogenously might be stored and processed differently within the muscle, at least in the short-term. One possibility which remains to be explored is the extent to which s-nitrosothiols may be formed in muscle and contribute as NO precursors.\textsuperscript{26} A strength of the study design, which involved unilateral knee extension exercise, was that we were able to study changes in [NO\textsubscript{2}] in both the exercised and unexercised (control) legs following NO\textsubscript{3} ingestion. These measurements showed that \textsuperscript{15}N labeled muscle [NO\textsubscript{3}] was decreased only in the exercised leg, thereby ruling out the effects of elapsed time and revealing that contractile activity is important in changes to muscle [NO\textsubscript{3}].

Total skeletal muscle [NO\textsubscript{2}] did not change during exercise following either NO\textsubscript{3} or placebo ingestion, consistent with our previous study,\textsuperscript{15} but in contrast to previous studies in rats in which it was increased following exercise.\textsuperscript{13} Considered alongside the observation that muscle [NO\textsubscript{2}] was not increased following NO\textsubscript{3} ingestion, it appears that human muscle [NO\textsubscript{2}] remains relatively stable when challenged by dietary and exercise interventions. It might be speculated that this apparent immutability is important in preserving muscle NO homeostasis. However, it should be pointed out that the measurement of NO\textsubscript{2} in muscle is technically challenging and that small changes in [NO\textsubscript{2}] might not be detected using current methodological approaches.\textsuperscript{27} Following placebo but not NO\textsubscript{3} ingestion, there was a significant increase in unlabeled muscle [NO\textsubscript{2}] post-exercise compared to pre-exercise. This raises the possibility that NOS-mediated NO synthesis might have been blunted by NO\textsubscript{3} ingestion.\textsuperscript{25}

Total plasma [NO\textsubscript{2}] decreased slightly during exercise following NO\textsubscript{3} but not placebo ingestion. Similar to the pattern observed in muscle, there was a significant reduction in \textsuperscript{15}N labeled plasma [NO\textsubscript{3}] but also a small increase in unlabeled plasma [NO\textsubscript{3}]. Total plasma [NO\textsubscript{2}] also decreased during exercise following NO\textsubscript{3} ingestion. These results are consistent with earlier studies, which reported that plasma [NO\textsubscript{2}] declines significantly during intense cycle or running exercise, especially following...
Supplementation. A surprising finding in the present study was that the decrease in total plasma [NO3−] was caused by a fall in unlabeled [NO2−] since 15N labeled [NO2−] was unchanged. Whether these differences in labeled and unlabeled plasma [NO3−] and [NO2−] dynamics reflect movements between body compartments, interplay between the NOS and NO3−-NO2−-NO pathways, or have functional significance is uncertain at the present time. However, the use of a stable isotope tracer in the present study provided important insight into the balance between temporal changes in basal/endogenous and exogenously-supplied NO3− (and NO2−) in blood and muscle following NO3− ingestion.

3.3 | Exercise performance

Study participants completed a 5-min all-out test involving 60 intermittent (3-s contraction, 2-s rest) isometric MVCs of the knee extensors. The muscle was electrically stimulated on the first, 15th, 30th, 45th, and 60th contraction. This protocol enables measurement of dynamic changes in torque development as well as assessment of the contribution of central and peripheral factors to fatigue development.

We found no differences between NIT and PLA for voluntary activation or the potentiated doublet response, suggesting that central and peripheral fatigue development was similar between the conditions. Moreover, there was no difference in the end-test torque or impulse above end-test torque. However, the mean torque produced during muscular contractions over the first 90s of the test (i.e. first 18 contractions) was significantly greater for NIT compared to PLA. These results contrast with one study, which reported no significant effect of acute NO3− ingestion on indices of central or peripheral fatigue or on time-to-task-failure during intermittent isometric knee extension exercise, but are consistent with another study, which found that 5 days of NO3− supplementation reduced the rate of muscle fatigue development and extended the time-to-task-failure during dynamic knee extension exercise. Recent meta-analyses have highlighted the potential for dietary NO3− supplementation to enhance skeletal muscle force or power production during high-intensity exercise. Acute NO3− ingestion has been reported to result in a ~5% increase in peak power during isokinetic dynamometry whereas chronic (5–6 days) NO3− supplementation improved performance during a 30-s cycle sprint and 5–20-m running sprints. Dietary NO3− supplementation also appears to enhance the intrinsic contractile properties of human skeletal muscle, as evidenced by increased force production at low frequencies of electrical stimulation, an increased rate of force development or a lower metabolic cost of force production.

A possible explanation for the improvement in muscle torque production over the first 90s of the 5-min all-out exercise test is differences in calcium (Ca2+) handling or sensitivity between the NIT and PLA conditions. Hernandez et al. reported that, in mouse fast-twitch (but not slow-twitch) muscle, 7 days of NO3− treatment elevated myoplasmic free [Ca2+] and increased contractile force at ≤50 Hz of electrical stimulation. The present study required participants to make a series of maximum isometric contractions of their knee extensors for 5 min. In this type of all-out exercise, recruitment of muscle fibers will be near maximal at the outset with a high proportional contribution from type II (fast-twitch) fibers to torque generation. Therefore, specific effects of ingested NO3− on Ca2+ handling and contractility in type II muscle fibers might be hypothesized to play a role in the improved torque generation we observed in NIT compared to PLA over the first 90s of the exercise test. This hypothesis would also be consistent with a recent report that fast-twitch muscle has a greater [NO2−] than slow-twitch muscle. Practically, our results suggest that athletes competing in sports which require maximal rates of force production and acceleration from the start to overcome inertia or for tactical advantage may benefit from dietary NO3− supplementation. However, the increased capacity for force generation afforded by NO3− ingestion may also be beneficial in continuous or intermittent high-intensity activities in which physical effort is more evenly distributed. Further studies are required to investigate the specific effects of NO3− ingestion on fast-twitch fiber NO3− and NO2− content, Ca2+ handling and contractility.

An important finding in the present study was the significant correlation between both the pre-exercise 15N labeled muscle [NO3−] and the magnitude of the fall in 15N labeled muscle [NO3−] during exercise and the improvement in muscle torque production during the first 90s of the 5-min all-out test. Indeed, this is the first study to show that pre- and in-exercise muscle [NO3−] dynamics following acute dietary NO3− supplementation are linked to enhanced exercise performance. Interestingly, there was no correlation between changes in plasma [NO3−] or [NO2−] and exercise performance, indicating that local muscular rather than systemic elevation of NO3− may be important in the ergogenic effects of dietary NO3− supplementation. The original finding that acutely elevated muscle [NO3−] is associated with greater muscle torque production may have implications for improving functional outcomes in a wide range of human populations, from those that may be compromised by senescence or disease through to elite athletes.
It was not the purpose of this study to attempt to quantify the proportional distribution of the ingested NO$_3^-$ in muscle, plasma, saliva, urine and, by subtraction, other tissues. Doing so would require assessment of the participants’ body composition and skeletal muscle mass, hematocrit and plasma volume, and total urinary output, as well as assumptions regarding uniformity of distribution of NO$_3^-$ in all skeletal muscle based on measurements made in the *vastus lateralis*. It should be noted, however, that some portion of the ingested NO$_3^-$ would have entered organs such as the liver, heart, kidney and brain. Given the relatively high concentration of NO$_3^-$ in skeletal muscle and the fact that skeletal muscle mass may represent as much as 50% of total body mass, it is clear that skeletal muscle may represent an important storage site for NO$_3^-$.

In this study, we used a K$^{15}$NO$_3$ tracer to address our experimental hypotheses. However, dietary NO$_3^-$ supplementation most often occurs in the form of beetroot juice, and it is unclear if or how our results might have differed if NO$_3^-$-rich beetroot juice had been consumed. We employed acute NO$_3^-$ ingestion in the present study, and it is also unclear how longer-term NO$_3^-$ supplementation (over several days or weeks) might have influenced our findings. Finally, our study was conducted in a young male population and further studies are required to establish whether females and older people respond similarly.

4.1 | Participants

Inclusion criteria were ostensibly healthy males and females, free of cardiovascular, respiratory, metabolic and musculoskeletal disorders, or having any contraindication to maximal exercise. Exclusion criteria included use of antibacterial mouthwash or tongue scrapers, dietary supplements, blood pressure medication, and tobacco smoking. Although females were eligible and welcome to participate in this study, only males volunteered ($n = 10$; age: $23 \pm 4$ years, height: $1.80 \pm 0.07$ m, body mass: $87.7 \pm 8.5$ kg, BMI: $26.4 \pm 1.0$ kg/m$^2$).

4.2 | Experimental design

In a randomized, crossover study, participants were allocated to one of two conditions, which involved the consumption of a K$^{15}$NO$_3$ tracer (NIT group: 12.8 mmol, ~1300 mg NO$_3^-$; 1 g/L, 99% $^{15}$N, CK Isotopes, Desford, UK) or an equimolar potassium chloride (KCl) placebo (PLA: negligible nitrate) (Figure 6). To minimize the number of muscle biopsies taken from participants and because we have previously shown that no changes occur in skeletal muscle [NO$_3^-$] following the ingestion of PLA,$^{15,16}$ there were five biopsies during the NIT condition and two biopsies (pre and post-exercise) during the PLA condition. Participants were informed that they would provide a different number of muscle tissue samples between the two conditions, but this would be balanced across the group such that a larger number of biopsies did not indicate which condition was NIT and which condition was PLA. To control for potential variations in the participants’ habitual diets, a 3-day dietary control period preceded the experimental visits. These consisted of an initial 2-day period in which participants were provided with a list of foodstuffs containing high NO$_3^-$ and NO$_2^-$ and asked

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**FIGURE 6** Schematic of experimental protocol including timings of sample collection.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIT</td>
<td>07:30 (Arrival)</td>
<td>Pre-baseline sample</td>
</tr>
<tr>
<td>NIT</td>
<td>08:30 (~0.5 h) (Supp ingestion)</td>
<td>Pre-dose sample</td>
</tr>
<tr>
<td>PLA</td>
<td>09:00 (1-h)</td>
<td>Pre-dose sample</td>
</tr>
<tr>
<td>NIT</td>
<td>10:00 (2-h)</td>
<td>Pre-exercise sample</td>
</tr>
<tr>
<td>NIT</td>
<td>11:00 (3-h) (Pre-exercise)</td>
<td>Post-exercise sample</td>
</tr>
<tr>
<td>NIT</td>
<td>12:00 (~3.5h) (Post-exercise)</td>
<td>Post-dose sample</td>
</tr>
</tbody>
</table>

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**MATERIALS AND METHODS**

This study was approved by the Sport and Health Sciences Ethics Committee (University of Exeter) in line with the principles of the Declaration of Helsinki. Once the associated risks and benefits of the investigation were thoroughly explained, all participants that enrolled in the study provided written consent before taking part in any experimental procedures.
to abstain from consuming them, and a final day during which they were provided with a controlled diet containing ~25–30 mg NO$_3^-$−. The experimental visits were separated by a minimum of 7 days and a maximum of 10 days. This duration has been shown to be sufficient for muscle [NO$_3^-$] to return to baseline values following NO$_3^-$− supplementation.$^{16}$

On each experimental visit, participants arrived at the laboratory in a rested and fasted state at 07:30 a.m. Upon arrival, participants were asked a series of questions regarding their adherence to the prescribed diet. An initial urine sample was collected after which participants were seated on a bed and were requested to refrain from excessive movement for the remainder of the sample collection period. A saliva sample was then collected, and an intravenous cannula was inserted into the antecubital fossa and a blood sample was collected. Preparations for the muscle biopsies were completed after the initial blood samples had been processed and the initial muscle tissue sample was then collected. Following the biopsy, a low NO$_3^-$− breakfast (two slices of toast with 10 g butter) was provided at ~08:50 a.m., and at 09:00 a.m. the K$^{15}$NO$_3$ tracer was ingested in the form of a 140 ml drink. The drink was created on the morning of the visit by dissolving 1.31 g of either the K$^{15}$NO$_3$ tracer (NIT, 12.8 mmol, ~1300 mg NO$_3^-$−) or KCl (PLA, negligible nitrate), weighed using analytical grade scales, in 140 ml deionized water. The container was vigorously shaken to ensure that the powder had fully dissolved before the participant ingested the drink. The NIT and PLA drinks were indistinguishable in appearance, smell and taste.

All subsequent biological samples were collected in relation to the supplement ingestion time. The collection time of the muscle tissue extraction was at 1- and 3-h post-supplement ingestion, with saliva and blood collected before, and urine collected after, the muscle sampling was completed. In the NIT condition, the 3-h biopsy also served as the “pre-exercise” biopsy and, following the exercise protocol, two biopsies were taken (one from each leg) within 20 s of the cessation of exercise (Figure 6). In the PLA condition, only two biopsies were taken: one at 3 h post-supplement ingestion (i.e., pre-exercise) and a second following the completion of the exercise protocol (i.e., post-exercise). A maximum of two biopsies were taken from each incision site.

### 4.3 Exercise protocol

The exercise protocol involved the participants performing a series of 60 intermittent isometric maximal voluntary contractions (MVCs) of the knee extensors with muscle stimulation (i.e., the “5-min all-out test” first described by Burnley$^{20}$). The protocol was unilateral such that one leg served as the exercise leg and the other served as a control.

Participants were seated in the chair of a Biodex System 3 isokinetic dynamometer (Biodex Medical Systems, Shirley, NY), which was calibrated according to the manufacturer’s instructions. The dominant leg was attached to the lever arm of the dynamometer with the seating position adjusted to ensure that the lateral epicondyle of the right femur was centered with the axis of rotation of the lever arm. The subjects sat with relative hip and knee angles of 85° and 90°, respectively (full extension being 0°), determined using an inclinometer. The lower leg was firmly attached to the lever arm above the ankle using a padded Velcro strap, and straps secured firmly across the waist and shoulders prevented extraneous movement during the isometric contractions. Carbon rubber electrodes (12×10 cm, EMS Physio, Oxfordshire, UK) coated in conductive gel were placed on the anterior thigh and secured using micropore tape. The cathode was placed on the midline of the thigh at ~30% of thigh length measured in the seated position from the anterior superior iliac spine to the superior border of the patella, while the anode was positioned over the femoral artery. A constant-current, variable voltage stimulator (Digitimer DS7AH, Welwyn Garden City, UK) was used to deliver a series of stimuli between which the anode was adjusted until an optimal electrical stimulus or “saturation point” was achieved. Once the site had been confirmed, the anode was secured using micropore tape and a 0.5 kg sandbag was placed on top. The voltage was set at 400 V and the current was then incrementally increased until there was a plateau in the evoked twitch. The current was then increased to 130% of the plateau current and this was subsequently used to deliver a doublet stimuli (100-μs pulses, 10-ms interval). Participants were familiarized with the experimental setup and exercise protocol prior to the commencement of the main experiment.

Following a warm-up involving a series of submaximal contractions (three at 50%, two at 75% and one at 90% of estimated MVC), participants completed three MVCs, each lasting 3 s and separated by 60 s rest. The second of these contractions was performed with electrical stimulation of the muscle. Subjects were given a countdown followed by very strong verbal encouragement to maximize torque. At 1.5 s into the contraction a doublet was delivered, subjects were instructed to stop the contraction after 3 s, and another doublet was delivered 1 s after the end of the contraction. Following the third MVC, subjects rested for 10 min.

Participants then completed a 5-min all-out test involving 60 intermittent isometric MVCs (3-s contraction and 2-s rest). During the test the subjects were strongly...
encouraged to maximize torque during each contraction but were not informed of the elapsed time or the number of contractions remaining. The muscle electrical stimulator was triggered to deliver a doublet on the first, 15th, 30th, 45th, and 60th contraction. Stimuli were delivered 1.5 s into each of these contractions, and 1 s after each contraction.

The torque data were analyzed using Spike 2 software (Cambridge Electronic Design Ltd., Cambridge, UK). Briefly, a horizontal cursor was set on the torque axis at 15 N.m to exclude any potential false triggers and the start and end of each contraction was defined as the intersection between two additional vertical cursors with the horizontal cursor. The Spike 2 software then determined peak torque and mean torque for each 3-s contraction for both tests (i.e., NIT and PLA). The peak mean torque was defined as the 3-s contraction that yielded the highest mean torque. For any given time bin (such as over the whole 5 min test or across the first 90-s), the peak torque and the mean torque produced during each contraction was determined and used to calculate the mean peak torque and the mean mean torque, respectively. The torque impulse was calculated as the area under the torque-time curve. The potentiated doublet torque was calculated as the peak torque achieved following the doublet stimuli between contractions, and superimposed doublet torque was calculated as the increment in torque immediately following the stimuli during contraction. The end-test torque, which provides an estimate of the critical torque (CT), was operationally defined as the mean of the last six contractions in the 5-min all-out test. Voluntary activation was determined using the twitch interpolation technique.51

4.4 | Sample collection

Muscle tissue samples were collected from the vastus lateralis muscle using a modified percutaneous Bergström needle procedure adapted for manual vacuum.32 A venous cannula (20 G Insyte-WTM cannula; Becton Dickinson, Madrid, Spain) inserted in the antecubital fossa was used to draw blood samples for the determination of plasma 

\[ \text{NO}_3^- \] and \[ \text{NO}_2^- \] in the antecubital fossa was used a 20 G Insyte-WTM cannula (Becton Dickinson, NJ) and centrifuged at 3300 g for 7 min at 4°C. The extracted plasma was then placed in liquid nitrogen before being stored in a −80°C freezer. Two-min saliva collection periods were employed to enable participants to generate sufficient saliva before expelling it into a 30-mL universal tube (Thermo Scientific™ Sterilin™; Massachusetts, USA). The saliva was then aliquoted and placed in liquid nitrogen before being stored at −80°C. Urine samples were collected in separate containers (Kartell™, Milan, Italy) and aliquoted into micro-centrifuge tubes for storage.

4.5 | Measurement of total \[ \text{NO}_3^- \] and \[ \text{NO}_2^- \] in biological samples

Ozone-based gas-phase chemiluminescence was used to quantify \[ \text{NO}_3^- \] and \[ \text{NO}_2^- \] in the biological samples collected during the study using helium as the carrier gas (Sievers 280i Nitric Oxide Analyzer, GE Analytical Instruments, Boulder, CO, USA). The initial step for sample processing prior to injection into the NO analyzer was to add methanol to the plasma, urine and saliva samples (1:2 ratio by volume). These were thoroughly vortexed, left to incubate at room temperature for 30 min and subsequently centrifuged at 4°C and 11 000 g for 5 min. The supernatant was then collected and injected into the analyzer configuration. Vanadium chloride or tri-iodide solution was used for nitrate or nitrite analysis, respectively. Muscle samples were weighed and processed to ensure consistency between the sample sizes (~40–60 mg), a NO2− preservation solution was added (K2Fe(CN)6, N-ethylmaleimide, water, Nonidet P-40), and then the samples underwent a series of homogenization steps using a bead homogenizer (Bertin Minilys, Bertin Instruments, France). Muscle \[ \text{NO}_3^- \] and \[ \text{NO}_2^- \] was then determined using the methods described by Park et al.27 For all tissues, part of the supernatant was processed for UPLC–MS/MS analysis (see below) and the rest was used for NOA.

4.6 | Determination of 15NO3− or 15NO2− proportion by UPLC–MS/MS

To measure NO3− content by UPLC–MS/MS, NO3− in all samples were first reduced to NO2− enzymatically by bacterial nitrate reductase from Aspergillus niger (N7265, Sigma-Aldrich, St. Louis, MO, USA) as previously described with some modification. Briefly, the sample (20 or 2 μl for urine) was mixed with nitrate reductase (0.1 U/mL) and NADPH (100 μM) and incubated for 2 h at room temperature. Then NO2− in samples were derivatized with 2,3-diaminonaphthalene (DAN, D2757, Sigma-Aldrich, 5 mM) for 30 min at 37°C to yield 2,3-naphthotriazoe (NAT). NaOH (58 mM) was added to terminate the reaction. For measuring NO2− content only, samples (50 μl) were directly subjected to DAN derivatization. High-performance liquid chromatography (HPLC) grade solvents and LC-MS modifiers were purchased from Sigma-Aldrich (St. Louis, MO, USA). Detection and quantification were achieved by UPLC–MS/MS utilizing a Thermo Scientific Vanquish UPLC with a Thermo
Scientific Altit triple quadrupole mass spectrometer, heated electrospray ionization (HESI-II) in positive ion mode (3500 V). 50 μl of sample was mixed with 200 μl of acetonitrile (ACN), vortexed for 5 min and then centrifuged at 4°C and 17000 g for 15 min. The supernatant was transferred to an LC-MS vial for analysis. Injection volume was 1 μl. A Waters Cortecs T3 column, 2.1 × 100 mm, 1.6 μm column was maintained at 35°C. Solvent A: H2O with 0.1% formic acid (FA) and Solvent B: ACN with 0.1% FA. The flow rate was 250 μl/min, the gradient was 25% B at 0 min for 0.25 min, increasing to 65% B at 5 min, further increased to 90% B at 5.5 min, remained at 90% B until 7.5 min, and then decreased to 25% B at 8 min. The total running time was 10 min. Samples were analyzed in triplicate. Quantitation of 14NAT and 15NAT were based on multiple reaction monitoring (MRM) transitions m/z, 170.062 → 115.042 and 171.062 → 115.042, respectively. The result was based on the percentage ratio of 15NAT/(14NAT + 15NAT).

4.7 Statistical analysis

The Statistical Package for Social Scientists (SPSS Version 28, SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the data. Full sets of data were available for muscle, saliva and urine (n = 10) whereas, due to technical error, data were available for plasma for nine participants. Two-way repeated measures ANOVAs were used to determine differences in [NO3−] and [NO2−] in muscle, plasma, saliva and urine across time (pre-exercise and post-exercise) and condition (PLA and NIT). A separate one-way repeated measures ANOVA was also run for the NIT condition for the exercise component of the study, which included a control and experimental leg. Where appropriate, significant main and interaction effects were analyzed further using least significant difference (LSD) post hoc tests. Relationships between variables were evaluated using Pearson’s product moment correlation coefficients. The alpha level to denote statistical significance was p < 0.05. All results are expressed as mean ± standard deviation (SD).

5 Conclusion

We used a stable isotope tracer (K15NO3) to show that acute inorganic NO3− ingestion increases plasma, salivary and urinary [NO3−] and that the ingested NO3− is also taken up rapidly by skeletal muscle. We also show that, following its elevation by dietary supplementation, muscle [NO3−] falls substantially during exercise, and that both the pre-exercise muscle [NO3−] and the magnitude of its decrease during exercise are correlated with muscle torque production during maximal voluntary contractions of the knee extensors. These results provide new insight into the regulation of muscle contractile function by mechanisms related to NO generation and suggest that dietary NO3− supplementation may provide a means of enhancing human muscular performance.

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CONFLICT OF INTEREST

Alan N. Schechter is listed as a co-inventor on several patents issued to the National Institutes of Health for the use of nitrite salts for the treatment of cardiovascular diseases. He receives royalties based on NIH licensing of these patents for clinical development but no other compensation. The other authors declare that they have no conflicts of interest.

ORCID

Anni Vanhatalo https://orcid.org/0000-0003-2546-5667
Peter J. Walter https://orcid.org/0000-0001-8571-398X
Andrew M. Jones https://orcid.org/0000-0002-2082-1709

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